Fungal Genetics and Biology 47 (2010) 869–878

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Conflict between reproductive gene trees and species phylogeny among heterothallic and pseudohomothallic members of the filamentous ascomycete genus *Neurospora*

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ARTICLE INFO

Article history:
Received 9 April 2010
Accepted 17 June 2010
Available online 1 July 2010

Keywords:
Ascomycota
Evolution
Heterothallism
Mating type
Pheromone receptor
Pseudohomothallism

ABSTRACT

In this study, we investigated the genealogies of genes important for sexual identity, i.e. mating-type (*mat*) and pheromone-receptor (*pre*) genes, among heterothallic and pseudohomothallic taxa of *Neurospora*. The resulting genealogies were compared with the species phylogeny derived from non-coding sequences. We found conflicting topologies between the reproductive genealogies and the species phylogeny, and these conflicts were supported by both node support analyses and likelihood tests on the relative fit of datasets to alternative phylogenetic hypotheses. We argue that reproductive genes are more permeable to gene flow, i.e. are more often introgressed between species of *Neurospora*, than other parts of the genome. Certain conflicts between the species phylogeny and both *mat* genealogies were observed, suggesting that the two mating-type idiomorphs were selectively introgressed into a species from a single ancestral source. Taken together, the results presented here highlight complex evolutionary trajectories of reproductive genes in the fungal kingdom, which may be of importance for reproductive behavior in natural populations.

1. Introduction

Sexual reproduction is a fundamental biological process among higher eukaryotes. In recent years, the genes involved in sexual reproduction in eukaryotes have gained significant attention in evolutionary biology, largely because they show evidence of strong positive selection and may impact the establishment of reproductive barriers that lead to speciation (Palumbi, 2009; Swanson and Vacquier, 2002), but also due to the general connection between the evolution of reproductive genes and the reproductive behavior of organisms (Paoletti et al., 2006; Walters and Harrison, 2008; Wik et al., 2008).

Pioneer work on the basis for genetic control of mating has emerged from the fungal kingdom (Heitman et al., 2007). However, data on evolutionary constraints and inheritance of genes involved in reproduction is primarily available from the animal and plant kingdoms (Clark et al., 2006; Palumbi, 2009), and data from the fungi on the topic has just recently become available (Aguileta et al., 2010; Karlsson et al., 2008; Wik et al., 2008). The filamentous ascomycete genus *Neurospora* has a long history as a model organism (Davis and Perkins, 2002) and is an excellent model system for the study of evolution, speciation and population biology (Dettman et al., 2003a,b; Karlsson et al., 2008; Turner et al., 2001; Wik et al., 2008). This genus has representatives of the most common sexual strategies known to exist in the fungal kingdom, i.e. heterothallism, pseudohomothallism, and homothallism.

Heterothallic *Neurospora* species have a bipolar mating system; individuals are of either mating type *A* or *a* and these mating types, unlike those of certain yeast species, do not switch (Perkins, 1987). Isolates of different mating types must meet for sexual reproduction to occur. This process includes cell fusion, nuclear pairing, nuclear fusion and meiosis, resulting in four *mat a* and four *mat A* haploid ascospores contained in an ascus (Coppin et al., 1997). The pseudohomothallic taxa *Neurospora tetrasperma* develops four-spored asci in which the ascospores contain nuclei of both mating types, resulting in self-fertile heterokaryons (Raju and Perkins, 1994), and homothallic taxa reproduce sexually in an

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doi:10.1016/j.fgb.2010.06.008

autonomous fashion, and often carry genes of both mating types within each haploid nucleus (Coppin et al., 1997). The relationship between species of heterothallic and pseudohomothallic taxa of *Neurospora* has been critically examined by both phylogenetic and reproductive compatibility criteria (Dettman et al., 2003a, 2006; Menkis et al., 2009; Villalta et al., 2009), and no molecular evidence in support of recent interspecific gene flow or the existence of true hybrids has been found.

Sexual reproduction in heterothallic *Neurospora* is controlled by the mating-type (*mat*) locus and its downstream targets (Shiu and Glass, 2000). The *mat* locus in *Neurospora* is a small and specialized region of the genome (Glass et al., 1990; Staben and Yanofsky, 1990). The *mat* sequences are not alleles in the classical sense, because although present at a homologous genomic location, they are completely dissimilar, and consequently they were proposed by Metzenberg and Glass (1990) to be termed idiomorphs. In contrast to the idiomorphs, the regions flanking the *mat* locus are found to be homologous (Glass et al., 1990; Staben and Yanofsky, 1990).

In heterothallic *Neurospora* the two mating-type idiomorphs, known as *mat a* and *mat A*, contain genes encoding different transcription factors controlling both mating in the sexual cycle and function in establishing heterokaryon incompatibility in the vegetative cycle (Glass et al., 1988). One open reading frame (ORF), *mat a-1*, was identified in the model species *Neurospora crassa* for controlling mating in *mat a* strains (Staben and Yanofsky, 1990). In the idiomorph of *mat A* strains three ORFs, *mat A-1*, *mat A-2*, and *mat A-3*, have been identified. Although *mat A-1* is considered to be of major importance for the control of sexual development, *mat A-2* and *mat A-3* appear to play an important supporting role (Ferreira et al., 1996; Glass et al., 1990). The *mat* genes in *Neurospora* control the downstream targets governing the whole expression cascade of mating-type-specific expressed genes, including the pheromone-receptor genes *pre-1* and *pre-2*. These latter two genes are not genetically linked to the *mat* genes and exist in the genomes of strains of both mating types, however *pre-1* is predominantly expressed in *mat A* strains whereas the expression of *pre-2* is induced in *mat a* strains (Karlsson et al., 2008; Kim and Borkovich, 2006). Comparatively low levels of between species nucleotide identity have been observed for the *mat* genes as well as the *pre* genes in filamentous ascomycetes (Poggeler, 1999; Poggeler and Kuck, 2001) and this divergence was recently confirmed to be partly driven by positive selection (i.e. significant support for positive selection was detected for a few codons) for all four *mat* genes and *pre-1* in heterothallic *Neurospora*. However, the driving force behind the positive selection is still unknown and a background rate of neutral evolution also contributed to an increased divergence between species (Karlsson et al., 2008; Wik et al., 2008).

In this study, we analyzed the full sequences of the *mat* and *pre* genes from heterothallic and pseudohomothallic taxa of *Neurospora*. The focus of this study is exclusively on heterothallic and pseudohomothallic species, since they form a well-supported monophyletic clade and has been challenged for numerous phylogenetic studies of species relationship (Cai et al., 2006; Dettman et al., 2001, 2003a; Poggeler, 1999). The main finding of this study is that the reproductive gene trees differ from the species tree generated from a concatenated dataset of four non-coding autosomal loci, and we interpret the differences as the result of ancient and recent introgression between the species.

2. Materials and methods

2.1. Fungal material

A set of 35 strains belonging to ten phylogenetic taxa of heterothallic *Neurospora* and the pseudohomothallic *N. tetrasperma* were used in this study (Table 1). All strains were obtained from the Fungal Genetics Stock Center (FGSC; Department of Microbiology, University of Kansas Medical Center, Kansas City, KS). The taxa formerly known as PS1, PS2, and PS3 (Dettman et al., 2003a), are referred to by their new epithets, *Neurospora hispaniola*, *Neurospora metzenbergii*, and *Neurospora perkinsii* (Villalta et al., 2009), respectively, throughout this paper.

2.2. Selection of loci

Four mating-type genes (*mat a-1*, *mat A-1*, *mat A-2* and *mat A-3*) and two pheromone-receptor genes (*pre-1* and *pre-2*) were investigated in this study. Both the complete coding sequences and introns were included in the analyses. These genes are here referred to as reproductive genes. The microsatellite-flanking loci DMG, TMI, TML, and QML were used to infer a species phylogeny, as in Dettman et al. (2003a). Finally, partial regions of genes with assumed functions independent of sexual reproduction in *Neurospora* were selected for comparative purposes; actin (*act*), β-subunit of tubulin (*Bml*), glyceraldehyde 3-phosphate dehydrogenase (*ccg-7*), protein kinase C homologue (*pkc*) and translational elongation factor EF-1 α (*tef-1*). These genes are located on different chromosomes in the *N. crassa* genome (http://www.broadinstitute.org/annotation/genome/neurospora/) and in this paper they are referred to as non-reproductive genes.

2.3. DNA extraction and PCR amplification

Strains were grown in minimal medium broth (Vogel, 1964) with 1% sucrose for 3 days, after which nucleic acids were extracted as previously described (Johannesson and Stenlid, 1999). Primers for the reproductive genes were described earlier (Karlsson et al., 2008; Wik et al., 2008) and for the non-reproductive genes in Johannesson et al. (2000), and in Glass and Donaldson (1995). All PCR reactions were performed using Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN, USA) following Wik et al. (2008).

2.4. DNA sequencing

PCR products were purified with either EXOsap-IT (USB, Cleveland, OH) or Exo (Exonuclease I, New England BioLabs, Ipswich, MA) and SAP (Shrimp Alkaline Phosphatase, USB) according to manufacturer's recommendations. The sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The products of the sequencing reactions were cleaned using BigDye XTerminator Purification Kit (Applied Biosystems). Sequencing was partially performed by Macrogen Inc., Seoul, Korea, and at the Department of Evolutionary Biology, Uppsala University, Sweden, utilizing an ABI3730XL (Applied Biosystems). Raw sequence data were analyzed using the Sequencher 4.1.4 software (Gene Codes Corporation, Ann Arbor, MI) and BioEdit version 7.05.2 (Hall, 1999). Nucleotide sequences were aligned using the program MUSCLE (Edgar, 2004) and the alignments were adjusted manually. All sequence data are submitted to TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S10703).

2.5. Phylogenetic analysis

Phylogenetic analyses were performed on the following datasets: nucleotide alignments for *mat a-1* (representing 20 strains), *mat A-1* (19 strains), *mat A-2* (17 strains), *mat A-3* (18 strains), *pre-1* (33 strains), and *pre-2* (35 strains) (Table 1). Partition homogeneity tests were performed, as described below, for all possible pair-wise combinations of *mat A-1*, *mat A-2*, and *mat A-3*, and

Table 1
Fungal strains of *Neurospora* used in this study, their FGSC number, geographic location, mating type, and the reproductive genes analyzed.

Taxa	FGSC number	Geographic location	Mating type	*mat* genes analyzed	*pre* genes analyzed
Heterothallic taxa					
N. crassa (NcA[a])	8848	Caribbean Basin	a	a1	1, 2
N. crassa (NcA[a])	8876	Caribbean Basin	a	a1	1, 2
N. crassa (NcA[a])	8900	Africa	A	A1, A2, A3	1, 2
N. crassa (NcA[a])	8856	Africa	A	A1, A2, A3	1, 2
N. crassa (NcB[a])	8830	Africa	A	A1, A2, A3	1, 2
N. crassa (NcB[a])	8771	India	a	a1	1, 2
N. crassa (NcB[a])	8772	India	a	a1	1, 2
N. crassa (NcC[a])	8858	India	A	A1, A2, A3	2
N. crassa (NcC[a])	8860	India	a	a1	1, 2
N. crassa (NcC[a])	8863	India	a	a1	1, 2
N. crassa (NcC[a])	8867	India	A	A1, A3	2
N. hispaniola	8815	Caribbean Basin	a	a1	1, 2
N. hispaniola	8817	Caribbean Basin	A	A1, A2, A3	1, 2
N. hispaniola	8818	Caribbean Basin	A	A1, A2, A3	1, 2
N. intermedia (NiA[a])	8776	Caribbean Basin	a	a1	1, 2
N. intermedia (NiA[a])	8786	Caribbean Basin	A	A1, A2, A3	1, 2
N. intermedia (NiA[a])	8869	India	a	a1	1, 2
N. intermedia (NiA[a])	8901	Africa	A	A1, A2, A3	1, 2
N. intermedia (NiA[a])	8841	Africa	a	a1	1
N. intermedia (NiB[a])	8768	East Asia	a	a1	1, 2
N. intermedia (NiB[a])	8840	Africa	A	A1, A2, A3	1, 2
N. intermedia (NiB[a])	8844	East Asia	A	A1, A2, A3	1, 2
N. intermedia (NiB[a])	8778	East Asia	a	a1	–
N. metzenbergii	8847	Caribbean Basin	A	A1, A2, A3	–
N. metzenbergii	8853	Caribbean Basin	a	a1	1, 2
N. metzenbergii	8880	Africa	A	A1, A2, A3	1, 2
N. metzenbergii	8881	Africa	a	a1	1, 2
N. perkinsii	8835	Africa	a	a1	1, 2
N. perkinsii	8838	Africa	A	A1, A2, A3	1, 2
N. perkinsii	8842	Africa	a	a1	1, 2
N. sitophila	412		a	a1	2
N. sitophila	1135	Panama	A	A1	1, 2
N. sitophila	8770	India	A	A1, A2, A3	–
PS6 (*N. discreta*)[b]	8827	Africa	a	a1	1, 2
PS7 (*N. discreta*)[b]	8780	Caribbean Basin	A	A1, A2, A3	1, 2
Pseudohomothallic taxa					
N. tetrasperma[c]	3998	Raleigh, NC, USA	A	A1, A2, A3	1, 2
N. tetrasperma[c]	4245	Raleigh, NC, USA	a	a1	1, 2
N. tetrasperma[d]	9030	Franklin, LA, USA	A	A1, A2, A3	1, 2
N. tetrasperma[d]	9031	Franklin, LA, USA	a	a1	1, 2

[a] Intraspecific subgroups designated by Dettman et al. (2003).
[b] Phylogenetic species designated by Dettman et al. (2006a).
[c] Phylogenetic lineage 3 of the *N. tetrasperma* species complex (Menkis et al., 2009).
[d] Phylogenetic lineage 8 of the *N. tetrasperma* species complex (Menkis et al., 2009).

confirmed that these data sets were not in significant conflict ($p < 0.005$). Consequently they were concatenated into one data set (*mat A*). Genealogies for the non-reproductive genes were generated, including at least one strain of each taxon. The nucleotide sequences of four neutral markers, DMG, TMI, TML, and QML, were used to construct the species tree (TreeBASE matrix accession numbers: M1574 (Dettman et al., 2003a) and M4488 (Menkis et al., 2009)), including all strains of Table 1.

We used the Akaike information criteria in ModelTest 3.06 (Posada and Crandall, 1998) to estimate the following substitution models as best-fit for the data: model TrN + I for *mat a-1*, *mat A*, *act*, *ccg-7*, *pkc* and *tef-1*; HKY + G for *pre-1*; TVM + I + G for *pre-2*; K81uf + G for *Bml* and GTR + I + G for the microsatellite flanking regions (TMI, DMG, TML and QMA).

Phylogenetic analyses of sequences were done with PAUP* 4.0b10 (Swofford, 2003) using maximum likelihood (ML) default heuristic search settings and the best-fit model of sequence evolution. Gaps were treated as missing data in the analyses. PS6 and PS7 (formerly belonging to *N. discreta*) sequences were used for outgroup rooting of the phylogenies (Dettman et al., 2003a, 2006).

Both bootstrap and Bayesian analyses were performed to reveal branch support for each phylogeny. ML bootstrap values were obtained with the program PHYML 2.4.4 (Guindon and Gascuel, 2003), using 1000 replicates and the best-fit model of sequence evolution. This program was used instead of PAUP due to its greater speed. The threshold for significance of ML bootstrap branch support values was set to 75%, which have been shown likely to correspond to confidence levels of at least 95% (Hillis and Bull, 1993). In addition, Bayesian Markov chain Monte Carlo (MCMC) analyses were performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Since MrBayes does not implement all models available in ModelTest, we used the program MrModelTest (Nylander, 2004) and AIC to select the best model for sequence evolution: HKY + G for *mat a-1* and *pre-1*; HKY + I for *mat A*; GTR + I + G for *pre-2* and for the microsatellite flanking regions (TMI, DMG, TML and QMA); GTR + I for *act*, *ccg-7*; GTR + G for *Bml* and *efa* and HKY + I + G for *pkc*. Two separate runs were carried out with four Markov chains, each initiated from a random tree. The Markov chains were run for one million generations and the trees were sampled every 100 generations. Default cold and heated chain parameters were used. At the end of each run, the sampling of the posterior distribution was considered to be adequate if the average standard deviation of split frequencies was <0.01. MCMC runs were summarized using the "sump" and "sumt" commands

in MrBayes and 2500 trees (25%) from each run were discarded as "burn-in". The threshold for significant Bayesian node support was considered 0.95 (Larget and Simon, 1999). The trees were visualized with TreeView (Page, 1996).

2.6. Partition homogeneity testing

In order to determine whether phylogenetic signals of the different datasets were in significant conflict, we used the partition homogeneity (PH) test (Farris et al., 1995a, 1995b) in PAUP* 4.0b (Swofford, 2003), with 1000 replicates and the heuristic general search option. This test randomly shuffles informative sites among pairs of loci, and if the datasets are compatible, shuffling of sites between the loci should not produce summed tree lengths greater than that produced by the observed data. We tested the datasets between reproductive and non-reproductive coding gene datasets, in all possible pair-wise combinations. In addition, PH tests were performed between the datasets of all coding genes and the microsatellite flanking regions. For all loci, we used one strain per taxon in the test. For *pre-2* we did not include the strains showing intraspecific divergence, i.e. FGSC 8842 (*N. perkinsii*) and FGSC 8901 (*N. intermedia* A). The null hypothesis of congruence was rejected if $p < 0.05$.

2.7. Hypothesis testing

Shimodaira–Hasegawa (SH) tests were performed upon the datasets generated from the reproductive genes (*mat a*, *mat A*, *pre-1*, and *pre-2*) and the dataset of the concatenated sequences of the flanking regions of microsatellite markers (TMI, DMG, TML and QMA) in order to compare the fit of the datasets to alternative phylogenetic hypotheses. SH tests were accomplished by comparing trees generated using RAxML (Stamatakis, 2006) according to ML criteria, under topological constraints corresponding to the incongruencies observed between the reproductive gene trees and the species tree, and by comparing the likelihood of the unconstrained (maximum likelihood) tree with that of the constraint tree(s). This test was performed only between different reproductive gene and microsatellite datasets, since the non-reproductive gene trees were poorly resolved.

The following conflicts between the reproductive gene tree topologies and species tree topology were tested:

1. Monophyly of *N. crassa* subgroups A–C (NcA, NcB, and NcC) as seen in the species tree compared to the polyphyly observed in both *mat* genealogies and *pre-2* genealogy.
2. The conflicting placement of *N. metzenbergii* between the species tree and both *mat* genealogies.
3. The conflicting placement of *N. tetrasperma* between the species tree and both *mat* genealogies.
4. The different placement of *Neurospora sitophila* and *N. hispaniola* in the gene trees of *mat a* and *mat A*.
5. Monophyly of *N. intermedia* subgroups A and B (NiA and NiB) as seen in the species tree but not in *pre-1* and *pre-2* genealogies.
6. Monophyly of *N. perkinsii* as seen in the species tree compared to paraphyly observed in the *pre-2* genealogy.

Unconstrained and constrained phylogenetic trees were generated by ML heuristic search using RAxML. These trees were imported into PAUP* 4.0b10. To directly evaluate the support of the datasets for competing phylogenetic hypotheses, we used the Shimodaira–Hasegawa (SH) test to statistically evaluate the inferred best tree with alternative topologies, based on 1000 bootstrap replicates of RELL ("resampling estimated log-likelihood") as applied in PAUP*.

3. Results

3.1. Genealogies from reproductive and non-reproductive datasets

Two species trees, one for the *mat a* and one for the *mat A* strains were reconstructed from a concatenated dataset of the microsatellite flanking markers (TMI, DMG, TML, QMA) published previously (Dettman et al., 2003a; Menkis et al., 2009). These two species trees, together with the *mat a* and *mat A* genealogies are depicted in Fig. 1. The *pre-1* and *pre-2* genealogies are shown in Fig. 2, and the genealogies of the non-reproductive gene datasets are found in Supplementary information.

To investigate the possible impact of selection on the gene tree topologies, all codons that had one or more amino-acid substitution were excluded from phylogenetic analyses. The resulting trees displayed the same branching pattern for *mat a*, *mat A* and *pre-1*. For *pre-2*, the resulting genealogy was not highly resolved, but did not contradict the tree including all codons (data not shown).

3.2. Comparison between mat genealogies and species phylogeny

The *mat* genealogies and the species trees displayed different topologies. Three primary conflicts were detected, in which branching nodes were significantly supported (ML bootstrap of $\geqslant$75% and Bayesian node support of $\geqslant$0.95) in both alternative groupings (Fig. 1). First, in the species tree, *N. crassa* subgroups A–C (NcA, NcB and NcC) form a monophyletic group, in contrast to both of the *mat* genealogies, in which NcC forms a well-supported monophyletic group with *N. intermedia* subgroups A and B (NiA and NiB). We have sequenced the *mat A* genes from two additional strains of NcC (FGSC numbers 8859 and 8865) and these are identical, or almost identical, to the *mat A* sequences from the other NcC strains included in this study (data not shown), indicating that *mat* genes from all NcC strains show a close relationship to *N. intermedia*.

Second, the placement of *N. metzenbergii* and *N. tetrasperma* differs between the *mat* genealogies and the species tree. In the species trees, *N. metzenbergii* forms a separate branch not included into any of the other two well-supported ingroup clades, and *N. tetrasperma* forms a well-supported cluster together with *N. sitophila*, *N. hispaniola*, *N. perkinsii*, NcA, NcB and NcC. In the *mat a* genealogy, *N. metzenbergii* itself is paraphyletic, and clusters together with *N. perkinsii*, NcA, NcB, NcC, NiA, and NiB, and in the *mat A* genealogy, *N. metzenbergii* forms a monophyletic group together with *N. sitophila*, *N. hispaniola*, *N. perkinsii*, NcA and NcB. In the *mat A* genealogy, *N. tetrasperma* forms a sistergroup to the rest of the ingroup taxa, and in the *mat a* genealogy it forms a clade with *N. sitophila* and *N. hispaniola*, which together are the sister group of the remaining ingroup taxa (Fig. 1).

Finally, the *mat a* and *mat A* genealogies show contradictory placements of *N. sitophila* and *N. hispaniola* (Fig. 1). In the *mat a* genealogy, these two taxa form a well-supported monophyletic group with *N. tetrasperma*, whereas in the *mat A* genealogy they cluster together with all ingroup-taxa except *N. tetrasperma*.

3.3. Comparison between pre genealogies and species phylogeny

The *pre* genealogies and the species trees displayed different topologies, and three primary significantly supported conflicts were detected (Figs. 1 and 2). First, in the *pre-1* genealogy, NiA and NiB do not form a monophyletic group, as in the species tree; instead NiB clusters together with *N. metzenbergii* (Fig. 2). Second, in the *pre-2* genealogy, *N. crassa* subgroups A–C do not cluster in a monophyletic group, as they do in the species tree. Finally, in the *pre-2* genealogy we found two cases of within species variation:

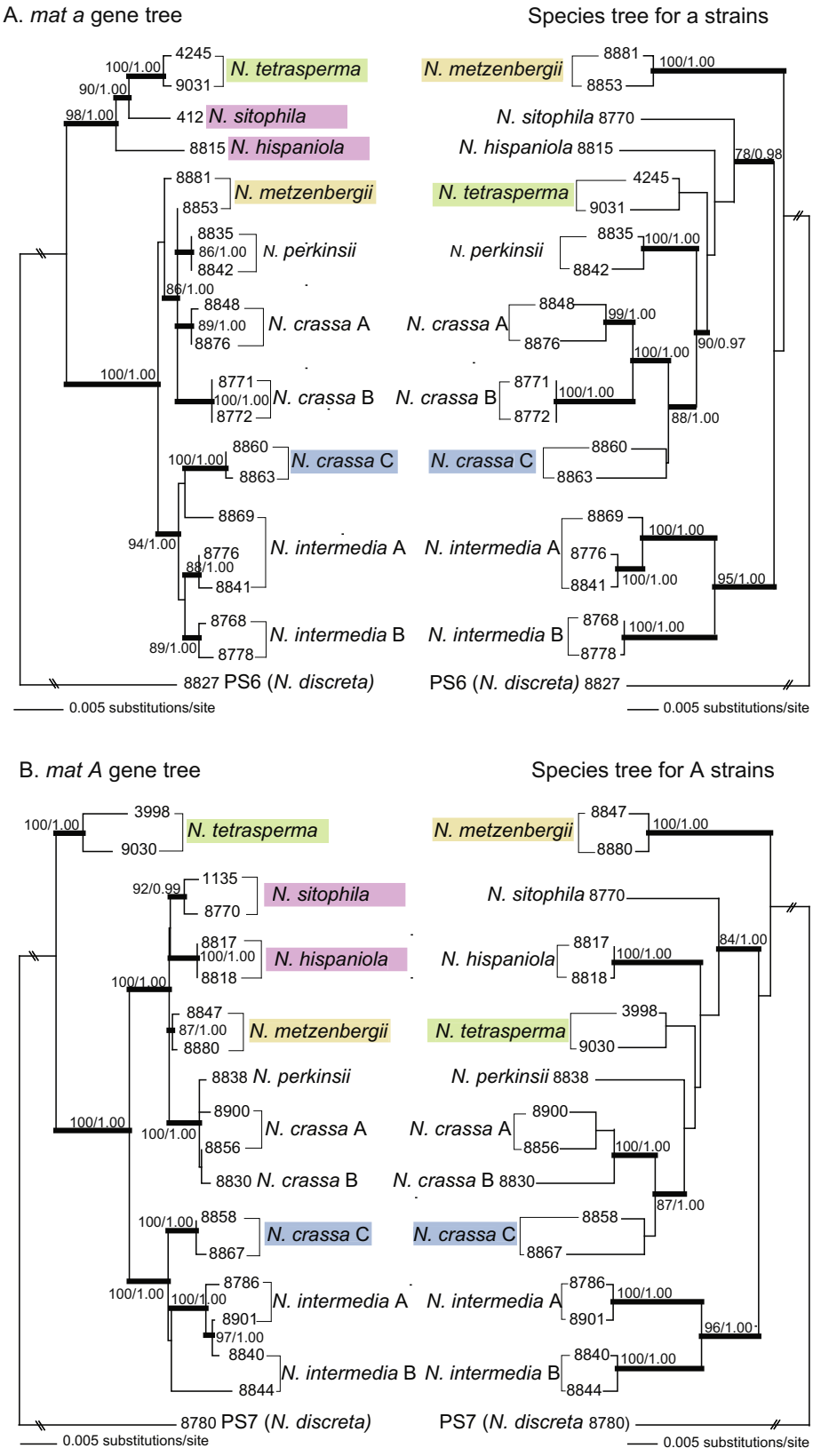

Fig. 1. Comparisons between the *mat* genealogies and species phylogeny for heterothallic and pseudohomothallic *Neurospora*. Topological comparison between *mat* genealogies and species tree for the taxa representing the *mat a* strains (A) and *mat A* strains (B). Node support values are shown as maximum likelihood bootstrap proportions/Bayesian posterior probabilities above nodes and are indicated as a thick line if the bootstrap support is ⩾75% and the Bayesian posterior probabilities ⩾0.95. Conflicting placements of taxa are highlighted.

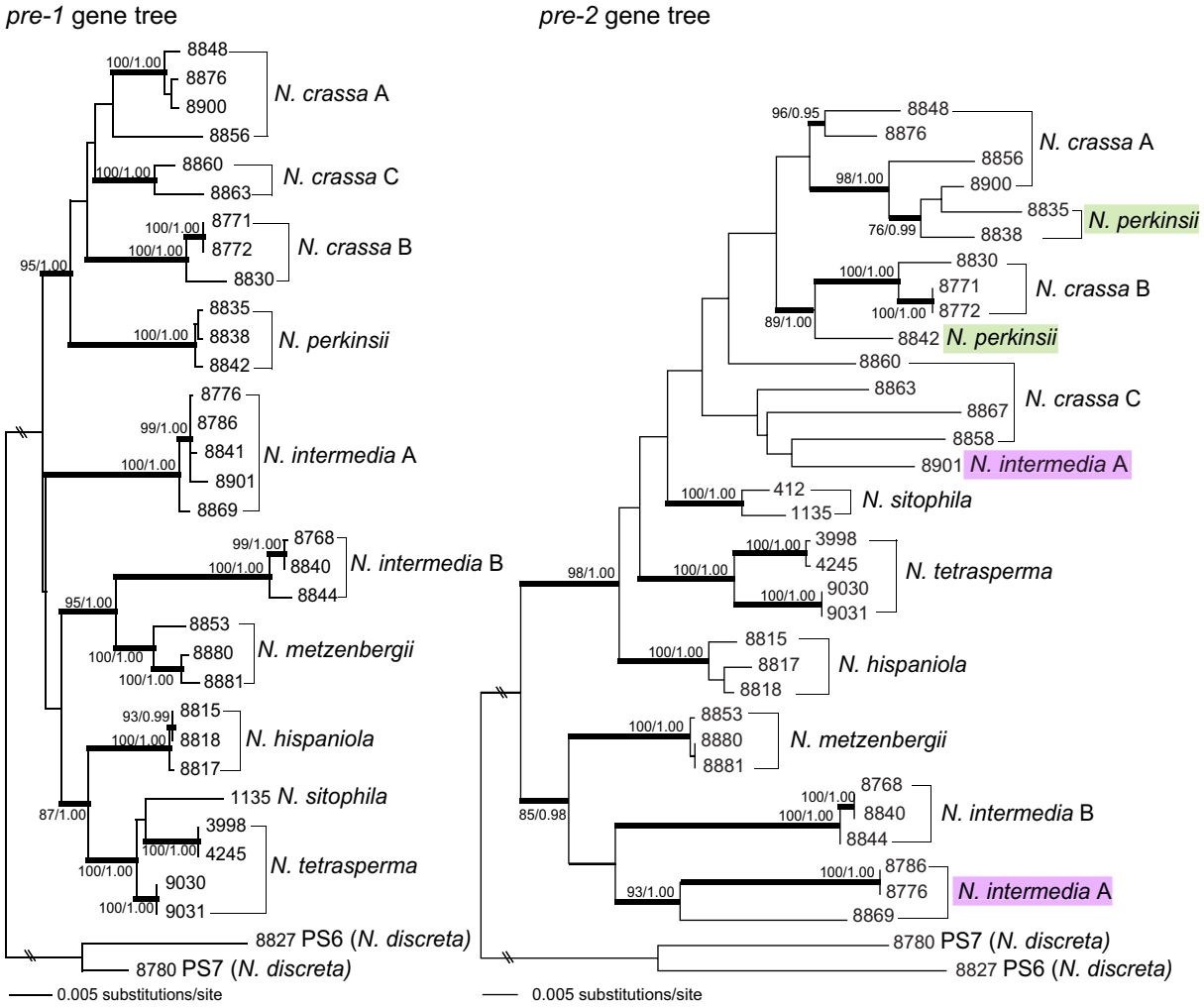

Fig. 2. The *pre-1* and *pre-2* genealogies of heterothallic and pseudohomothallic *Neurospora*. Node support values are shown as maximum likelihood bootstrap proportions/Bayesian posterior probabilities above nodes and are indicated as a thick line if the bootstrap support is ⩾75% and the Bayesian posterior probabilities ⩾0.95. Conflicting placements of taxa are highlighted.

one of the NiA strains (FGSC 8901) clusters together with NcC, separately from the other NiA and NiB strains included here, and in *N. perkinsii* strains FGSC 8835 and FGSC 8838 cluster together with NcA, separately from strain FGSC 8842 which clusters with NcB (Fig. 2).

3.4. Low resolution of the non-reproductive gene trees

The coding genes that are not known to be involved in mating are hereafter referred to as non-reproductive genes. Analyzed individually, the phylogenies of the non-reproductive genes showed little resolution (Supplementary Fig. 1a and b). In these five genealogies, only four well-supported branches were formed clustering different taxa together: first, for *ccg*-7 we found a branch supporting the clustering of *N. tetrasperma* and *N. sitophila*, while there is no support in the species tree for a sister relationship between these two taxa (Fig. 1). Second, for *pkc*, NcC and *N. perkinsii* clustered together. *N. crassa* (NcA, NcB and NcC) form a sister-clade with *N. perkinsii* in the species tree, and in the *mat* genealogies *N. perkinsii* cluster together with NcA and NcB (Fig. 1). In addition, in *tef-1*, a supported branch clustered one strain of *N. tetrasperma* together with *N. hispaniola*, thus *N. tetrasperma* showed multiple histories for this gene, and finally a well supported clade which groups the *N. perkinsii* with the majority of the *N. crassa* samples.

3.5. Partition homogeneity testing revealed conflicts between reproductive and non-reproductive genes

The results from PH-tests indicate that all non-reproductive gene datasets were in significant conflict with each of the *mat*-gene datasets ($p < 0.05$; Table 2). This pattern was also found for the microsatellite-flanking datasets, with the exception of the DMG microsatellite dataset which was not in conflict with any of the *mat* datasets. For *pre-1* and *pre-2*, we found 19 of the 36 pair-wise comparisons in conflict with the non-reproductive and microsatellite-flanking loci ($p < 0.05$; Table 2).

All investigated non-reproductive genes were found to be congruent ($p > 0.05$), with the exception of *tef-1*, which was incongruent with *act* and *pkc* (Table 3). The DMG microsatellite marker was congruent with all non-reproductive genes, which was also the case for TML with the exception of comparison with *act*. Conflicts were found between TMI, QMA and the majority of the non-reproductive genes (Table 3). These latter are the two most variable of the microsatellite-flanking loci (Dettman et al., 2003a).

3.6. Hypothesis testing

The results of the SH tests are summarized in Table 4. For both *mat a* and *mat A* gene datasets, unconstrained trees showed a

Table 2
P values from partition homogeneity testing performed pair-wise between the reproductive (horizontal) and non-reproductive (vertical) datasets. Two datasets were used for the non-reproductive datasets, one with *mat a* strains and one with *mat A* strains.

Locus	*mat a*	*mat A*	*pre-1 (a)*	*pre-1 (A)*	*pre-2 (a)*	*pre-2 (A)*
act	0.001	0.001	0.116	0.024	0.829	0.267
ccg-7	0.011	0.001	0.978	0.995	0.730	0.157
tef-1	0.002	0.001	0.009	0.006	0.237	0.001
pkc	0.001	0.001	0.001	0.990	0.030	0.001
Bml	0.014	0.008	0.562	0.374	0.332	0.396
DMG	0.083	1.000	0.943	0.996	0.996	0.998
TMI	0.001	0.001	0.005	0.004	0.010	0.001
TML	0.001	0.001	0.001	0.001	0.298	0.001
QMA	0.001	0.001	0.003	0.004	0.057	0.001
pre-1	0.001	0.001	n.a.	n.a.	0.023	0.001
pre-2	0.001	0.001	0.021	0.024	n.a.	n.a.

Abbreviations: mating type a (*mat a*); mating type A (*mat A*); pheromone receptor-1 (*pre-1*); pheromone receptor-2 (*pre-2*); actin (*act*); glyceraldehyde 3-phosphate dehydrogenease (*ccg-7*); translational elongation factor EF-1α (*tef-1*); protein kinase C homologue (*pkc*); β-subunit of tubulin (*Bml*); and microsatellite flanking regions (DMG, TMI, TML, and QMA).

significantly better fit for the data than the constrained trees enforcing monophyly of *N. crassa* subgroups A–C (conflict 1). The complementary SH test of this conflict was done for the dataset of the non-coding microsatellite-flanking loci, and noteworthy, the unconstrained tree did not fit the microsatellite-flanking data significantly better than a constrained tree enforcing NcC to form a monophyletic group with NiA and NiB, although the result was close to the threshold for significance ($p = 0.054$, Table 4).

The conflicting placement of *N. metzenbergii* and *N. tetrasperma* between the *mat* gene trees and the species phylogeny (conflicts 2 and 3, respectively) was tested. The clustering of *N. metzenbergii* with *N. sitophila*, *N. hispaniola*, *N. perkinsii*, NcA and NcB, as we see in the *mat A* genealogy, implies a significantly worse fit for the microsatellite-flanking dataset ($p = 0.006$). However, the clustering *N. tetrasperma*, *N. sitophila*, and *N. hispaniola*, testing the both conflicting placement of *N. metzenbergii* and *N. tetrasperma* between the *mat* genealogies and the species tree, was not a significant worse fit for the microsatellite-flanking dataset ($p = 0.070$), although this was closer to the significance-threshold than the constraint of *N. tetrasperma* being sistergroup to the rest of the ingroup ($p = 0.231$). Constraints imposing the conflicting placement of *N. sitophila* and *N. hispaniola* in the *mat a* and *mat A* genealogies (conflict 4) were supported in the hypothesis-testing (Table 4).

Furthermore, for *pre-1* the unconstrained tree provided a significant better fit for the data than the constrained tree ($p = 0.046$) which enforces monophyly of *N. intermedia* subgroups A and B as seen in the species tree (conflict 5). Finally, SH analyses were performed on the *pre-2* dataset to test the fit of the dataset to trees with enforced clades observed in the species tree: first, monophyly of *N. crassa* subgroups A–C (conflict 1); second, monophyly of *N.*

Table 3
P values from partition homogeneity testing performed pair-wise between datasets of the non-reproductive and microsatellite-flanking loci (DMG, TMI, TML, and QMA), for strains representing the *mat a* and *mat A* strains, respectively.

	act *mat a*/*mat A*	*ccg-7* *mat a*/*mat A*	*tef-1* *mat a*/*mat A*	*pkc* *mat a*/*mat A*	*Bml* *mat a*/*mat A*	DMG *mat a*/*mat A*	TMI *mat a*/*mat A*	TML *mat a*/*mat A*	QMA *mat a*/*mat A*
act	–/–	–/–	–/–	–	–/–	1.000/0.822	0.005/0.001	0.033/0.011	0.232/0.105
ccg-7	0.067/0.052	–/–	–/–	–	–/–	0.830/0.976	0.033/0.042	0.420/0.457	0.017/0.147
tef-1	0.014/0.019	0.906/0.991	–/–	–	–/–	0.976/1.000	0.001/0.005	0.156/0.145	0.001/0.002
pkc	0.478/0.077	0.421/0.405	0.015 /0.238	–	–/–	1.000/1.000	0.001/0.001	0.086/0.058	0.002/0.001
Bml	0.352/0.286	0.631/0.766	0.872/0.893	0.760/0.580	–/–	0.683/0.938	0.055/0.053	0.623/0.361	0.048/0.157

Abbreviations: actin (*act*), glyceraldehyde 3-phosphate dehydrogenease (*ccg-7*), translational elongation factor EF-1α (*tef-1*), protein kinase C homologue (*pkc*), and β-subunit of tubulin (*Bml*).

Table 4
Results of the Shimodaira–Hasegawa (SH) test comparing alternative topologies for constrained and unconstrained trees for sequences of heterothallic and pseudohomothallic *Neurospora* species.

Conflict	Phylogenetic constraint	Shimodaira–Hasegawa test			
		-ln L	Diff-ln L	*P*(<0.05)	Rejected
	Unconstrained *mat a* gene tree	2950	(best)		Best
1	(NcA, NcB, NcC)[A]	2979	29	0.003	Yes
4	Ntet alone sister to rest of ingroup[C]	2966	156	0.012	Yes
	Unconstrained *mat A* gene tree	7833	(best)		Best
1	(NcA, NcB, NcC)[A]	8054	221	0.000	Yes
4	(Ntet, Nhis, Nsit)[B]	7891	58	0.000	Yes
	Unconstrained *Neurospora* species tree	6532	(best)		Best
1	(NcC, NiA, NiB)[B,C]	6585	53	0.054	No
2	(Nsit, Nhis, Nmet, Nper, NcA, NcB)[C]	6595	63	0.006	Yes
3	Ntet sister to rest of ingroup[C]	6545	12	0.231	No
2,3	(Ntet, Nhis, Nsit)[B]	6555	22	0.070	No
	Unconstrained *pre-1* gene tree	7021	(best)		Best
5	(NiA, NiB)[A]	7048	27	0.046	Yes
	Unconstrained *pre-2* gene tree	6867	(best)		Best
1	(NcA, NcB, NcC)[A]	6997	130	0.000	Yes
5	(NiA, NiB)[A]	6954	87	0.001	Yes
6	(Nper)[A]	6912	45	0.021	Yes

Abbreviations: NcA = *N. crassa* subgroup A, NcB = *N. crassa* subgroup B, NcC = *N. crassa* subgroup C, NiA = *N. intermedia* subgroup A, NiB = *N. intermedia* subgroup B, Nhis = *N. hispaniola*, Nmet = *N. metzenbergii*, Nper = *N. perkinsii*, and Nsit = *N. sitophila*.
[A] As observed in the species tree.
[B] As observed in *mat a* gene tree.
[C] As observed in *mat A* gene tree.

intermedia subgroups A and B (conflict 5); and third, monophyly of *N. perkinsii* (conflict 6). In each of these cases the unconstrained tree provided a significantly better fit for the *pre-2* data than any of the constrained trees.

4. Discussion

The main finding of this study is that topologies for the reproductive gene trees (mating-type and pheromone-receptor genes) are in conflict with the species phylogeny among heterothallic and pseudohomothallic members of the genus *Neurospora*. Conflicting topologies between gene trees and species trees are well documented and can be signatures of evolutionary processes such as horizontal gene transfer (HGT), incomplete lineage sorting, hybridization or introgression (Degnan and Rosenberg, 2009). We argue that the conflicts observed here are due to reproductive genes being more permeable to gene flow, i.e. are more often introgressed between species of *Neurospora*, than other parts of the genome.

In general, interspecies gene transfer between prokaryotic organisms is well accepted and considered an important evolutionary force, while its occurrence and frequency between eukaryotes is still controversial (Gogarten, 2003). Accordingly, HGT is often suggested as an explanation for incongruencies between gene trees and species trees, but is not considered to be a common phenomenon in ascomycetes (Dujon, 2005). On the other hand, numerous instances of ancient introgression in other fungal species complexes have been described, for example in *Microbotryum violaceum* (Le Gac et al., 2007), *Epichloë* spp. (Schardl et al., 1997), and *Botrytis* spp. (Staats et al., 2005). Although interspecific laboratory crosses of heterothallic *Neurospora* have resulted in a small number of viable progeny, and a geographical potential to hybridize exists (e.g. both *N. crassa* subgroup C and *N. intermedia* subgroups A and B, have been collected in Asia (Dettman et al., 2003a)), there is no molecular evidence in support of recent interspecific gene flow or the existence of true hybrids of *Neurospora* (Dettman et al., 2003a, 2006; Menkis et al., 2009; Villalta et al., 2009). This background suggests that any introgression between species of *Neurospora* is primarily ancient.

Among the conflicts between the *mat* genealogies and the species tree, the placement of *Neurospora crassa* subgroup C (NcC) is the most striking. The placement of NcC differs between the species tree, in which it is monophyletic with the other two phylogenetic subgroups of *N. crassa,* and both *mat A* and *mat a* genealogies, in which it clusters together with the subgroups of *N. intermedia*. The finding of *N. crassa* and *N. intermedia* forming two biological species (Dettman et al., 2003b) further supports the conflict. We argue that this pattern is due to ancient introgression of *mat a* and *mat A* genes between species. The fact that both *mat a* and *mat A* genealogies show the same conflicts with the species tree is an important finding, and indicates that the two mating types introgressed from a single ancestral source, rather than independently from different ancestors. This pattern strongly suggests a non-random process of acquisition of the *mat*-alleles in the evolutionary history of the species.

Chance events during lineage sorting can act on most or all loci in the genome, and should also be considered as a possible explanation for the observed conflicts between the *mat* genealogies and the species phylogeny (Degnan and Rosenberg, 2009). By the comparison between the reproductive and the non-reproductive datasets we tested if the conflicts are inherent only to the reproductive genes or if it is a genome wide phenomenon. The results from the PH-tests indicate that the *mat* genes are in conflict with both the non-reproductive genes and the majority of the microsatellite-flanking loci, and thus, we suggest that the conflict is specific to the *mat* genes. The deviating result from DMG, which was the only non-reproductive dataset congruent with both *mat a* and *mat A*-datasets in the PH analyses, may be due to the low resolution of the DMG data: a high proportion of the phylogenetically informative characters studied by Dettman et al. (2003a) were indels, which were not included in our analysis.

In addition to the conflicts between *mat* genealogies and the *Neurospora* species phylogeny, we observed conflicting topologies between both *pre-1* and *pre-2* genealogies and the species tree. In *Pre-1*, it concerns entire subgroups, indicating that it might be caused by an event early in the history of *Neurospora pre* genes similar to that of the *mat* genes. However, in almost half of the pair-wise comparisons between *pre-1* and the non-reproductive and microsatellite-flanking loci, we found the datasets to be congruent. These results make it difficult to exclude stochastic processes, such as chance events during lineage sorting, as the explanation for the observed conflicts in the relationship between species in the *pre-1* gene and the species tree. The most striking conflicts between the *pre* genealogies and the species tree were due to intrataxon variation in NiA and *N. perkinsii* in the *pre-2* genealogy. In the PH tests, only one strain per species were included, thus any conflict involving intraspecific diversity would not be recognized, however, we found support for these conflicts with the SH test. The evolutionary basis for these latter mentioned conflicts might indicate an event such as hybridization, which occurred more recently.

Crucial for introgression through natural hybridization is that the recombinant offspring perform better than their parents in at least some habitats (Arnold, 1997). Interestingly, interspecies transfer of mating-type genes has previously been reported from the Dutch elm disease fungus *Ophiostoma ulmi* into O. *novo-ulmi* (Paoletti et al., 2006). The transfer of both *MAT-1* and *vic* (vegetative incompatibility) loci has been found to occur several times during the spread of *O. novo-ulmi* across the world, indicating that interspecies gene transfer can drive rapid evolution of an invasive eukaryotic organism to enable it to conquer new environments. The reason why an interspecies gene transfer of mating-type or pheromone-receptor genes would be advantageous for *N. crassa* subgroup C is obscure. One may speculate that the *N. crassa* C lineage became essentially asexual for a long period, as a result of a shift driven by either stochastic or selective processes. A previous report indicates that absence of outcrossing (by means of obligate selfing under homothallism, or asexuality) is likely to result in a genetic decay of the *mat* loci (Wik et al., 2008), and since asexuality is assumed to be an evolutionary dead end (Smith, 1986), the acquisition of *mat* genes from other outcrossing lineages could re-invigorate the genetic makeup of *mat* genes necessary for outcrossing.

Several authors have pointed out the connection between reproductive gene evolution and speciation, and one of the most basic questions in biology asks what types of genetic forces drive speciation (Noor and Feder, 2006). The general trend observed among fungal species is that mating type is not involved in sexual isolation (Giraud et al., 2008). However, data suggests that pheromone cell signaling components of the *Ustilago* a mating-type loci are involved in sexual incompatibility between the species (Bakkeren and Kronstad, 1996), and the mating-type regulated pheromonal signal systems has also been suggested to be important for reproductive isolation in *Neurospora* species (Karlsson et al., 2008). Traits important for reproductive isolation is expected to be less prone to introgression between species, and given this connection between reproductive protein evolution and speciation, it is noteworthy that reproductive proteins appear to introgress more easily than other genes in the genomes of heterothallic *Neurospora*, as we have shown in this study.

In ascomycetes, several studies have been using *mat* genes for estimating the species tree, and in many cases the topologies of

the *mat* genes correspond with other molecular markers, for example in the *Fusarium graminearum* clade (O'Donnell et al., 2004), the crucifer pathogenic *Leptosphaeria maculans* species complex (Voigt et al., 2005), and in *Pyrenophora teres*, the causal agent of barley "net blotch" disease (Rau et al., 2007). However, the present and previous studies on the evolution of mating-type genes in *Neurospora* (Wik et al., 2008) indicates that *mat* genes may have a complex evolutionary history and should be not be used as a neutral marker to infer species relationship of fungi.

In addition to the conflicts discussed above, we see indications of introgressions between *mat* genes of *N. metzenbergii*, *N. tetrasperma* and other taxa, resulting in conflicts between the *mat* genealogies, and between each of them and the species tree. This calls for a deeper analysis of species relationship and on the genome evolution of mating-type loci and mating-type chromosomes in *Neurospora*. One way to improve the resolution of the species tree is to increase the number of loci used in the study (e.g. Rokas et al., 2003), and in order to do so, we performed a phylogenetic analysis concatenating the microsatellite flanking regions and the non-reproductive datasets, which resulted in a phylogeny much like the tree presented by Dettman et al. (2003a): the only difference is a monophyletic group formed between the two *N. intermedia* subgroups A and B together with *N. metzenbergii*. This finding further questions the conflict found between *pre-1* and species phylogeny based only on microsatellite flanking regions.

With the increasing abundance of molecular data and the recognition that evolutionary trees from different genes often have conflicting branching patterns (Felsenstein, 1988; Hudson, 1983; Maddison, 1997; Nichols, 2001; Pamilo and Nei, 1988; Tajima, 1983), it is becoming increasingly feasible to implement new analytical and simulation tools to investigate the magnitude of this discordance under probabilistic models of how genetic lineages evolve across species (Degnan and Rosenberg, 2009). In future studies, we aim to disentangle the species phylogeny of heterothallic and pseudohomothallic *Neurospora* by applying the novel approaches in phylogenetics using coalescent models to infer species phylogenies from multilocus data, and thereby we will be able to more specifically estimate the relative significance of introgression and hybridization in natural populations of *Neurospora*.

5. Concluding remarks

Taken together, the results presented here highlight complex evolutionary trajectories of reproductive genes in the fungal kingdom, which may be of importance for reproductive behavior in natural populations. Although we are not able to propose a specific model to explain the incongruencies between reproductive gene trees and species phylogeny, our findings contribute to a deeper understanding of the relative importance of interspecific gene transfer in eukaryotes, and in particular the evolutionary forces shaping the genomes of heterothallic and pseudohomothallic *Neurospora*.

Acknowledgments

We thank John W. Taylor for valuable comments on the project. We wish to thank anonymous referees for their thoughtful suggestions. Financial support to HJ from the Swedish Research Council, is greatly acknowledged. JES was supported by a postdoctoral fellowship from the Miller Institute for Basic Research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.06.008.

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